Defective Ristocetin-Induced Platelet Aggregation in von Willebrand's Disease and its Correction by Factor VIII

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ABSTRACT The antibiotic ristocetin, in concentrations of 1.0–1.5 mg/ml, aggregated normal platelets in citrated platelet-rich plasma by a mechanism in which the release reaction played only a minor role. Platelet aggregation by ristocetin in a concentration of 1.2 mg/ml was absent or markedly decreased in 10 patients with von Willebrand's disease. Lesser degrees of abnormality were obtained with a concentration of 1.5 mg/ml. The magnitude of the defect in ristocetin-induced platelet aggregation correlated well with the degree of abnormality of the bleeding time and the levels of antihemophilic factor (AHF, VIII$_{AHF}$) procoagulant activity. In all patients, the defect in ristocetin-induced platelet aggregation was corrected in vitro by normal plasma. Correction was also obtained with a fraction of normal cryoprecipitate that eluted in the void volume with VIII$_{AHF}$ after chromatography on a gel that excludes molecules larger than 5×10^6. A similar fraction, devoid of VIII$_{AHF}$ activity, obtained from patients with von Willebrand's disease had no corrective effect, but fractions obtained from patients with hemophilia were just as effective as those obtained from normal subjects. The correction activity of plasma and partially purified factor VIII was inhibited by a rabbit antibody to human factor VIII but not by a human antibody against VIII$_{AHF}$ procoagulant activity. The studies provide further evidence that patients with von Willebrand's disease are deficient in a plasma factor that is necessary for normal platelet function. The activity of this factor appears to be associated with factor VIII but is unrelated to VIII$_{AHF}$ procoagulant activity.

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INTRODUCTION

Patients with von Willebrand's disease have a dual defect in their hemostatic mechanism. They are deficient in factor VIII procoagulant activity (antihemophilic factor, AHF,[1] VIII$_{AHF}$)[2] and also have a prolonged bleeding time (1). The cause of the latter is unknown, but the poor retention of platelets in glass bead filters in patients with this disorder (1, 2) suggests a possible defect in the ability of platelets to function adequately during the primary arrest of bleeding. Previous studies indicate that the impaired platelet function in this disorder may not be related to an inherent platelet defect but rather to the deficiency of a plasma factor that is required for normal platelet function. Thus, the abnormal platelet retention can be corrected in vitro by normal plasma (3) or cryoprecipitate (4), and transfusions of these substances may also shorten the bleeding time (4–6). Recent studies suggest that these corrective effects are associated with the factor VIII molecule. For example, studies by Bouma et al. showed the factor in normal plasma that corrects the platelet retention defect elutes in the void volume with VIII$_{AHF}$ after chromatography on agarose gels that exclude molecules larger than 2×10^6 (7), and we have confirmed these findings (8). This corrective material, devoid of VIII$_{AHF}$,

[1] *Abbreviations used in this paper:* AHF, antihemophilic factor; HMW, high molecular weight; LDH, lactic dehydrogenase; PPP, platelet-poor plasma; PRP, platelet-rich plasma; VWF, von Willebrand factor; VIII$_{AGN}$, factor VIII antigen; VIII$_{AHF}$, AHF procoagulant activity; VIII$_{VWF}$, VWF associated with factor VIII molecule.

[2] In this paper, antihemophilic factor (AHF) procoagulant activity will be referred to as VIII$_{AHF}$. The term factor VIII is used to refer to the presumed molecular species in plasma on which VIII$_{AHF}$ is located.

was also present in fractions that eluted in the void volume after chromatography of hemophilic cryoprecipitate (7, 8) but was not found after chromatography of cryoprecipitate from patients with von Willebrand's disease (8).

Howard and Firkin have recently presented further evidence that platelet function is defective in von Willebrand's disease. They found that the addition of the antibiotic ristocetin to normal platelet-rich plasma results in platelet aggregation but that aggregation was decreased in two out of three patients with von Willebrand's disease (9). We present herein studies on the mechanism that may be involved in the aggregation of platelets by ristocetin, the results of ristocetin-induced platelet aggregation obtained in 10 patients with von Willebrand's disease, and findings that demonstrate that the abnormality of platelet aggregation in this disorder can be specifically corrected by a factor present in both normal and hemophilic plasma, which appears to be associated with factor VIII.

METHODS

Subjects. 10 patients with the typical findings of von Willebrand's disease (1) were studied (Table I). These consisted of a prolonged bleeding time, decreased $VIII_{AHF}$ activity and decreased retention of platelets in glass bead filters, as determined by previously described methods (1, 4). In the present study, $VIII_{AHF}$ levels were expressed as units per 100 ml rather than as percent activity. 1 U of $VIII_{AHF}$ is the amount present in 1 ml of the pooled, normal plasma used as the reference standard. Control subjects were fifteen normal subjects of both sexes, ages 19–45. Other patients with bleeding disorders who were studied had Glanzmann's thrombasthenia (10), classical hemophilia, and storage-pool disease (11, 12). In the latter disorder, collagen-induced platelet aggregation is defective owning to a deficiency of the metabolically inactive storage pool of adenosine diphosphate which, following its release, is responsible for platelet aggregation. Except where the effect of aspirin was being specifically studied, all subjects were requested to abstain from ingestion of drugs for at least a week prior to testing their blood.

Buffers. Unless otherwise stated, the buffer used in the study was prepared by mixing 1 part of 0.25 M Tris,[3] pH 7.3 + 2 parts of 0.85% saline (tris-saline buffer).

Platelet aggregation studies. Venous blood was mixed with 1/10 volume of 3.2% sodium citrate in silicone-treated tubes and centrifuged at 1,500 *g* and 20°C for 3 min to obtain platelet-rich plasma (PRP). Platelet-poor plasma (PPP), obtained by centrifuging PRP for 30 min in an International PR-J centrifuge at 2,400 *g* and 4°C, was used as the blank in the platelet aggregation studies and to assess the corrective effects of plasma. Platelet aggregation was studied by stirring 1.9 ml of PRP + 0.08–0.10 ml of the aggregating agent in a dual channel aggregometer equipped with a no. 609 deep red filter (Payton Associates, Buffalo, N. Y.), and results were recorded on a Riken-Denshi recorder (Tokyo, Japan). Platelet aggregation was expressed as the percent change in optical density 6 min after addition of the aggregating agent (13). All studies were performed within 3 h of blood collection. A connective tissue suspension, prepared and stored as previously described (13), was used as a source of collagen. Adenosine-5′-diphosphate·[sodium trihydrate] (ADP) was obtained from Calbiochem, Los Angeles, Calif., and was added to PRP in a final concentration of 4 μM. Polylysine (poly-L-lysine) was obtained from Schwarz/Mann, Orangeburg, N. J., and concanavalin A was purchased from Pharmacia Fine Chemicals, Inc., Piscataway, N. J. (Lot no. 3059). Ristocetin was obtained as a white powder from Abbott Laboratories, North Chicago, Ill., and was dissolved in Tris-saline buffer on the day of studying platelet aggregation.

Platelet release reaction. The amount of ADP that was released from the platelets during aggregation was determined by a slight modification of a previously described method (12). 1.9 ml of PRP was stirred with 0.1 ml of the aggregating agent (ristocetin or collagen) in the aggregometer cuvette at 37°C. After varying time intervals, 0.1 ml of 0.125 M EDTA[4] was added, and the contents of the cuvette were centrifuged at 10,000 *g* and 4°C for 30 min. The platelet button was suspended in Tris-HCl buffer, pH 7.4 containing 4.8 mM EDTA. ADP in the supernate and platelet suspension was extracted with an equal volume of 96% ethanol containing 10 mM EDTA, and the amount of ADP was determined by the method of firefly luminescence (12). Release of platelet serotonin was studied by a slight modification of the method of Jerushalmy and Zucker (14). [^{14}C]Serotonin creatinine sulphate[5] (57 mCi/mmol) was obtained from Amersham/Searle Corp., Chicago, Ill., and was dissolved in 70% ethanol in a concentration of 2 mM and stored at −20°C. To study the release of platelet-bound [^{14}C]serotonin, 30 ml of PRP was first incubated with 0.015 ml of the [^{14}C]-serotonin solution (final concentration 1 μM) for 30 min, during which period 88–94% of the radioactivity was incorporated into the platelets. The PRP was then stirred with collagen or ristocetin, and at varying time intervals the contents of the cuvette were transferred to centrifuge tubes, immersed in ice water, and subsequently centrifuged at 12,000 *g* for 15 min. Radioactivity in the supernates was counted in a Tri-Carb liquid scintillation counter (Packard Instrument Co., Downers Grove, Ill.), and release of platelet-bound [^{14}C]serotonin was calculated as a percent of the amount initially present in the platelets, as described by Jerushalmy and Zucker (14). Release of platelet lactic dehydrogenase (LDH) was studied in a test system employing washed platelets, prepared as described elsewhere (15). In essence, platelets from EDTA platelet-rich plasma were washed twice with Tris-buffered EDTA, pH 7.3, once with Tris-saline, pH 7.3, and then packed by centrifugation in a calibrated McNaught tube and diluted with Tris-saline to a concentration of 1% (vol/vol). The suspension contained about 300,000 platelets/mm^3. The test system consisted of 1.0 ml of the platelet suspension + 0.6 ml Tris-saline buffer + 0.4 ml of PPP + 0.1 ml of collagen or ristocetin. The mixture was stirred in the aggregometer for varying time intervals, and the platelets were sedimented by centrifugation, suspended in Tris-saline buffer and disrupted by sonication for 2 min (Sonic Dismembrator, Artek Systems Corp., Farmingdale, N. Y.). LDH was measured in the supernate and platelet sonicate by the method of Wroblewski and LaDue (16), using reagents obtained from Sigma Chemical Co., St. Louis, Mo.

[3] Tris-(hydroxymethyl)aminomethane.

[4] Na_2 ethylenediaminetetracetate.

[5] 5-Hydroxytryptamine-3′-^{14}C creatinine sulphate.

TABLE I

Ristocetin-Induced Platelet Aggregation in von Willebrand's Disease

	Bleeding time	VIII$_{AHF}$	Platelet retention (Salzman)	Ristocetin-induced aggregation: Platelet-rich plasma (PRP) 1.2 mg/ml	PRP 1.5 mg/ml	PRP + normal PPP* 1.2 mg/ml	PRP + normal PPP* 1.5 mg/ml
	min	*U/100 ml*	%	%	%	%	%
von Willebrand's disease‡							
D. R.	>60	4	6	0	0	7	71
J. H.	>60	4	20	0	0	8	73
C. M.	>60	3	27	0	0	18	84
J. L.	40	30	10	0	11	13	46
A. S.	24	23	2	8	18	61	67
P. H.	20	9	—	13	19	72	83
J. W.	15	13	25	12	8	76	93
M. L.	7	49	8	10	45	27	75
L. C.	6	41	14	8	90	79	86
D. P.	6	38	10	40	89	74	90
Normal subjects§							
Mean value	3.4	97‖	56	80	89		
SD	1.1		13	9	3.5		
95% CL**	1.2–5.6	49–194	31–83	62–98	82–96		

* 1.4 ml of patient PRP + 0.5 ml of normal PPP (platelet-poor plasma).

‡ Bleeding time and VIII$_{AHF}$ values on patients were obtained on day of studying ristocetin-induced aggregation; platelet retention (Salzman) values were obtained on previous studies.

§ Values obtained for ristocetin-induced aggregation were obtained on 15 normal subjects in the present study. Other normal values are from previously published data (1).

‖ Geometric mean.

** Confidence limits.

Inhibitors of platelet aggregation. The following substances were tested: adenosine (obtained from Sigma Chemical Co.), Na_2 EDTA, KCN, 2-deoxy-D-glucose (Aldrich Chemical Co., Inc., Milwaukee, Wisc.) and sodium heparin (Upjohn Co., Kalamazoo, Mich.).

Treatment of plasma. PPP was incubated for 10 min at room temperature with 1/10 volume of $Al(OH)_3$ (Cutter Laboratories, Berkeley, Calif.) or with Celite 512, 30 mg/ml (Johns Manville, New York). PPP was also incubated at 37° for varying periods of time. Cryoprecipitate was prepared as described previously (4) and was resuspended in the initial plasma volume with Tris-saline buffer. Serum was prepared from PPP by incubating for 1 h at 37°C the following mixture: 1.8 ml PPP + 0.1 ml 8% kaolin + 0.1 ml lipid (Platelin, a chloroform extract of brain obtained from Warner-Chilcott Laboratories, Morris Plains, N. J.) + 0.1 ml 0.025 M $CaCl_2$.

Agarose gel chromatography. A glass column (2.5 × 60 mm) was packed with Bio-Gel 5M, 200 mesh (Bio-Rad Laboratories, Richmond, Calif.) to a gel height of 50 cm and equilibrated with a buffer containing one part of 0.253 M imidazole and nine parts of 0.85% saline. The buffer, adjusted with 1M HCl, gave a pH of 6.9 at 22°C and 7.3 at 4°C. Cryoprecipitate obtained from 40 ml of plasma was dissolved in 2.0 ml of supernatant plasma. This was applied to the top of the column, and the column was eluted with buffer by gravity flow, by means of a Mariotte flask, at a pressure head of 56 cm. All procedures were performed at 4°C. The flow rate was 0.16–0.19 ml/min, and column fractions of 2.3–2.7 ml were assayed for VIII$_{AHF}$-activity and protein (OD_{280}) as previously described (17). The corrective effects of the fractions on ristocetin-induced platelet aggregation were tested on the day of collection. The agarose gel used excludes molecules whose molecular weight is greater than 5×10^6, and the void volume of the column was determined with *Salmonella typhosa* lipopolysaccharide, mol wt 1.5×10^8 (Difco Laboratories, Detroit, Mich.).

Antibodies to factor VIII. A monospecific rabbit antibody to purified human factor VIII (rabbit anti-VIII), the properties of which have been described (18), was obtained from Dr. Leon Hoyer of the University of Connecticut School of Medicine. We also used plasma from a hemophiliac with an antibody to factor VIII (human anti-VIII). Incubation of 0.9 ml of normal human plasma with 0.1 ml of the rabbit anti-VIII for 30 min at 37°C reduced the VIII$_{AHF}$ activity from 110 to 7%, while a similar incubation with the human anti-VIII reduced the VIII$_{AHF}$ activity from 110 to 2.5%. The inhibitory effects of both human and rabbit antibodies were specific for factor VIII; incubation with plasma had no effect on any other clotting factors.

RESULTS

Ristocetin-induced aggregation of normal platelets. The addition of ristocetin to normal PRP resulted in im-

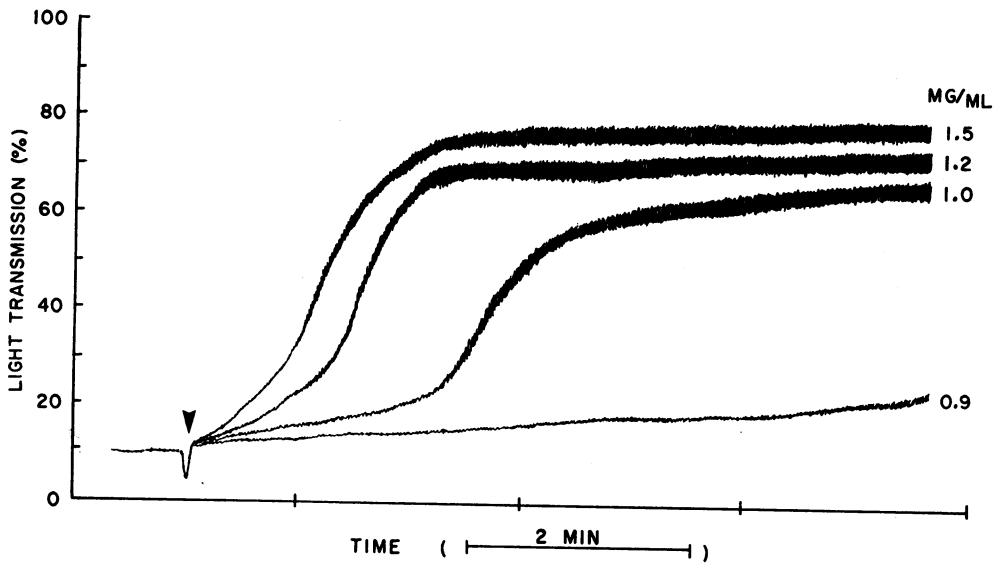

FIGURE 1 Ristocetin-induced platelet aggregation. Ristocetin, in the concentrations indicated, was added (▼) to normal platelet-rich plasma. Notice the rapid and transient decrease in transmission that occurred immediately after addition of ristocetin (see text). Platelet aggregation curves were drawn from the original tracings.

mediate platelet aggregation (Fig. 1). The lowest final concentration of ristocetin that produced some visible increase in light transmission on the aggregometer tracing varied but was generally between 0.8 and 1.0 mg/ml. With a concentration of 1.0–1.2 mg/ml, an initial slow rate of aggregation during the first 2–3 min was followed by a sudden and rapid increase in the slope of the aggregation curve. With higher concentrations of ristocetin, the rate of platelet aggregation was rapid from the onset, and separation of the aggregation curve into a slow and rapid phase was usually not apparent. A ristocetin concentration of 1.5 mg/ml produced a maximal amount of platelet aggregation in most normal subjects. An interesting finding was the rapid and transient decrease in transmission that occurred immediately after adding ristocetin. This dip (see Fig. 1) appears to be related in some way to plasma fibrinogen. It was also observed when ristocetin was added to normal PPP but did not occur with serum or with PPP from two patients with congenital afibrinogenemia. Addition of ristocetin to normal PPP in concentrations greater than 3 mg/ml resulted in the formation of a coagulum; this did not occur with plasma from two previously reported patients with congenital afibrinogenemia (19).

TABLE II
Effect of Inhibitors on Platelet Aggregation

Test substance*	Platelet aggregation: ADP (4 μM) 0‡	ADP (4 μM) 45‡	Ristocetin (1.2 mg/ml) 0‡	Ristocetin (1.2 mg/ml) 45‡
	%		%	
NaCl	84	80	89	88
Adenosine, 40 μM	23	20	25	20
Adenosine, 80 μM	8	0	8	13
KCN, 2 mM	71	80	94	78
2-deoxy-D-glucose(DG), 5 mM	81	65	91	87
KCN, 2 mM + DG, 5 mM	75	8	91	34
EDTA, 0.1%	0	0	45	32
KCN, 20 mM	40	20	0	4
Heparin, 1 U/ml	85	79	92	84
Heparin, 10 U/ml	84	87	86	95
NaCl	86	79	95	89

* Test substance (0.1 ml) was added to 1.9 ml of normal PRP (final concentration indicated). ADP or ristocetin was added either immediately after test substance (0 min incubation) or following an incubation period of 45 min.
‡ Incubation time at 37°C in minutes.

Effects of inhibitors on ristocetin-induced platelet aggregation. Various substances were tested for possible inhibitory effects on ristocetin-induced aggregation. For comparison, the effects on ADP-induced aggregation were studied in parallel. Results are shown in Table II. Both ristocetin and ADP-induced aggregation were inhibited to about the same degree by adenosine. Inhibition of respiration and glycolysis independently by 2 mM KCN and 5 mM 2-deoxy-D-glucose had little effect on either type of aggregation. However, simultaneous blockage of both metabolic pathways, obtained by incubating the PRP for 45 min with both inhibitors, drastically reduced the amount of platelet aggregation

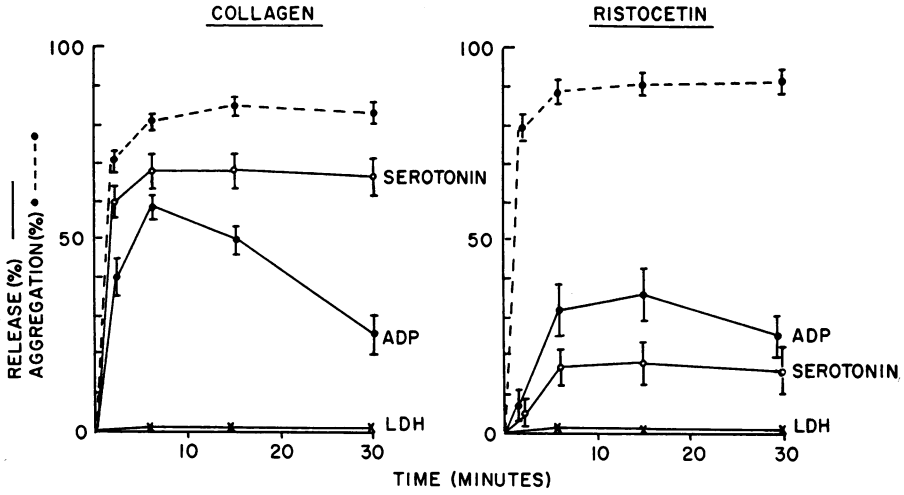

FIGURE 2 Release of platelet constituents during aggregation by collagen and ristocetin. Values shown for platelet aggregation (dashed lines) and release of ADP and platelet-bound [^{14}C]serotonin (as percent of the total amount) were obtained from studies on platelet-rich plasma of five normal subjects. Mean values ±SEM are shown. Release of platelet LDH was studied in a test system employing washed platelets and diluted plasma (see text) and the results of a typical experiment are shown. Note that with ristocetin (right), a significant amount of platelet aggregation (more than was obtained with collagen) at 2 min was not accompanied by the release reaction. The well-known association of collagen-induced aggregation with the release reaction is indicated by the results of the experiments shown on the left.

induced by ADP and, to a lesser degree, by ristocetin. EDTA, in a final concentration of 0.1%, completely eliminated ADP-induced aggregation but only partially blocked ristocetin-induced aggregation. The reverse was true of the inhibitory effects of a 20 mM concentration of KCN. Heparin did not inhibit platelet aggregation by either agent.

Release of platelet ADP, [^{14}C]serotonin and LDH by ristocetin and collagen. Parallel studies were performed on the release of ADP and platelet-bound [^{14}C]serotonin by concentrations of collagen and ristocetin that produced comparable platelet aggregation curves during the 30 min period in which the studies were performed (Fig. 2). With collagen, 60% of the platelet-bound [^{14}C]serotonin and 40% of the ADP was released at the end of 2 min. By contrast, only 5% of the serotonin and 7% of the platelet ADP was released by ristocetin during this same time interval, despite a marked degree (80%) of aggregation. Subsequently (at 6 min), 32% of the platelet ADP was released by ristocetin, compared with 62% released by collagen. The total amount (mean value) of platelet-bound [^{14}C]serotonin released by ristocetin did not exceed 18% during the 30 min study period, compared with a mean value of 68% release that occurred during collagen-induced aggregation. Neither collagen nor ristocetin-induced aggregation was associated with a release of platelet LDH.

Comparison of ristocetin-induced platelet aggregation in normal subjects and patients with von Willebrand's disease. Values obtained for ristocetin-induced aggregation are shown in Table I. In normal subjects, the amount of platelet aggregation produced by ristocetin in a concentration of 1.2 mg/ml was 80±9%; with a concentration of 1.5 mg/ml it was 89±3.5%. With the lower concentration, ristocetin-induced aggregation was either absent (four patients) or decreased in all 10 patients with von Willebrand's disease, as illustrated for patient C. M. in Fig. 3*A*. With the higher (1.5 mg/ml) concentration, aggregation was again absent or markedly decreased in seven of the patients. In one patient (M. L.), platelet aggregation was only moderately decreased (45%), and in two others (I. C. and D. P.), normal values of 90 and 89% were obtained. The latter three patients were the least severely affected among the group of 10 patients with von Willebrand's disease studied. They had the shortest bleeding times and highest VIII$_{AHF}$ values. In general, there was a good correlation between the degree of abnormality of ristocetin-induced aggregation and that of the bleeding time in the 10 patients studied.

Correction of the defect in platelet aggregation in von Willebrand's disease by normal plasma. The abnormal ristocetin-induced platelet aggregation in the PRP of all patients with von Willebrand's disease was corrected to varying degrees by the prior addition of normal, platelet-poor plasma as seen in Table I and illustrated in Fig. 3*B*. Except in the case of patient J. H., who had multiple antibodies to platelets, white cells and red cells,

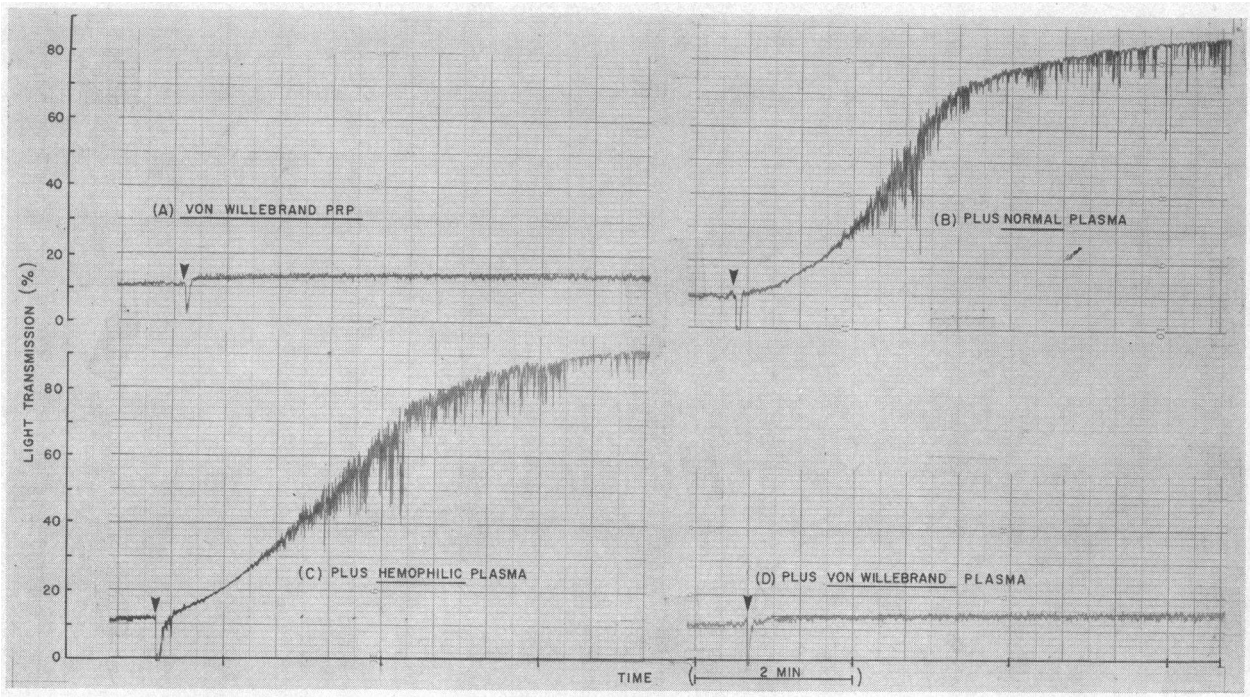

FIGURE 3 Ristocetin-induced platelet aggregation in von Willebrand's disease. Ristocetin, 1.5 mg/ml, was added to platelet-rich plasma (PRP) from (*A*) a patient (C. M.) with von Willebrand's disease, or the same PRP to which had been added 1/3 volume of platelet-poor plasma from (*B*) a normal subject, (*C*) a hemophiliac patient, (*D*) another patient (D. R.) with von Willebrand's disease. The actual tracings are shown.

the plasma of patients with von Willebrand's disease did not inhibit ristocetin-induced aggregation of normal platelets.

Properties of the plasma factor which corrects the platelet aggregation defect in von Willebrand's disease. Normal plasma was treated in various ways before assessing its corrective effect on the abnormal ristocetin-induced platelet aggregation in von Willebrand's disease (Table III). The activity of the correction factor was not absorbed by $Al(OH)_3$ or celite. Its activity in plasma was undiminished by freezing at $-60°C$ for a week but was significantly decreased after incubation at 37°C for 24 h. After slow thawing of frozen plasma, the factor was present in the cryoprecipitate but not in the supernate. The correction activity was present in serum prepared from plasma of normal and hemophiliac subjects but not from that of a patient with von Willebrand's disease. The corrective effect of hemophilic plasma on ristocetin-induced platelet aggregation was not accompanied by an increase in $VIII_{AHF}$ procoagulant activity.

Evidence that the correction factor is associated with factor VIII—chromatography of cryoprecipitate. Previous studies (see above) showed that the correction factor was present in cryoprecipitate. Cryoprecipitate obtained from normal plasma was chromatographed on Bio-Gel 5 M, and the fractions were analyzed for protein, $VIII_{AHF}$ and their ability to correct the abnormality of ristocetin-induced platelet aggregation of a patient with von Willebrand's disease. As seen in Fig. 4, $VIII_{AHF}$ appeared in the void volume, in association with a small amount of protein. These high molecular weight fractions that eluted in the void volume, and no others, also corrected the abnormality of ristocetin-induced platelet aggregation in a patient with severe von Willebrand's disease. By contrast a similar high molecular weight (HMW) fraction, obtained by chromatographing cryoprecipitate from a patient with von Willebrand's disease had only a slight corrective effect (Table IV). This suggests that patients with von Willebrand's disease are deficient in a high molecular weight factor, normally present in plasma, which is necessary for ristocetin-induced platelet aggregation. The correction factor activity of the high molecular weight fraction obtained from normal cryoprecipitate was destroyed by a rabbit antibody to human factor VIII (Table IV).

Evidence that the activity of the correction factor associated with factor VIII is unrelated to $VIII_{AHF}$ procoagulant activity. The results of several types of experiments indicate that the correction factor activity, although associated with the factor VIII molecule, is unrelated to the $VIII_{AHF}$ activity. The abnormality of ristocetin-induced platelet aggregation in von Willebrand's

disease was corrected by hemophilic plasma (Fig. 3C and Table IV) and by a high molecular weight (HMW) fraction, devoid of VIII$_{AHF}$ activity, that eluted in the void volume after chromatography of hemophilic cryoprecipitate on Bio-Gel 5M (Table IV). In addition, the corrective effect of normal plasma and of the HMW fraction derived therefrom (factor VIII) was not destroyed by a human anti-VIII that was a potent inhibitor to VIII$_{AHF}$ procoagulant activity (Table IV).

Ristocetin-induced platelet aggregation in other platelet disorders. Platelet aggregation curves, obtained with a ristocetin concentration of 1.2 mg/ml, are depicted in Fig. 5. Among congenital disorders, ristocetin-induced aggregation was decreased to a variable degree in two patients with Glanzmann's thrombasthenia. A subtle type of defect was present in patients with storage-pool disease and in normal subjects after aspirin ingestion. The initial slow phase of ristocetin-induced aggregation was normal. However, the slope of the more rapid phase was consistently decreased. Despite this lower rate of aggregation, the total amount of platelet aggregation at 6 min was the same as in normal subjects. Unlike the findings in von Willebrand's disease, none of the above abnormalities were corrected by the addition of normal plasma. Ristocetin-induced aggregation was normal in hemophilia.

Effects of other platelet-aggregating substances in von Willebrand's disease. Platelet aggregation by polylysine (0.05–0.10 mg/ml) was the same in patients with von Willebrand's disease as in normal subjects. The addition of concanavalin A in a final concentration of 3 mg/ml to both normal and von Willebrand PRP resulted in platelet aggregation after a lag period of 2–3 min.

TABLE III

Properties of the Plasma Factor that Corrects the Abnormalities of Ristocetin-Induced Aggregation in von Willebrand's Disease

Treated specimen no.	Source of plasma	Treatment	Ristocetin-induced aggregation*
			%
1	von Willebrand	None	0
2	Normal	None	84
3	Normal	$Al(OH)_3$	80
4	Normal	Celite	83
5	Normal	−60°C, 1 wk	80
6	Normal	37°, 24 h	20
7	Normal	Cryoprecipitate (cpt)	86
8	Normal	Supernate of cpt	11
9	Normal	None	84
10	Normal	37°C, 1 h	86
11	Normal	Serum (37°C, 1 h)	83
12	Hemophiliac	None	80
13	Hemophiliac	37°C, 1 h	77
14	Hemophiliac	Serum (37°C, 1 h)	83
15	von Willebrand‡	None	7
16	von Willebrand	37°C, 1 h	7
17	von Willebrand	Serum (37°C, 1 h)	5

* 1.4 ml of PRP from a patient with von Willebrand's disease +0.5 ml treated test plasma + ristocetin, final concentration 1.5 mg/ml.

‡ The VIII$_{AHF}$ value of the patient with von Willebrand's disease was 3 U/100 ml. The results of experiments 9–17 suggest that the corrective effect of serum is probably not owing to the nonspecific effects of procoagulants, but rather to the fact that the activity of the von Willebrand factor is not destroyed by coagulation.

DISCUSSION

Ristocetin is an antibiotic that is obtained from the actinomycete *Nocardia lurida* and is active against gram positive bacteria and mycobacteria (20, 21). Because of the high incidence of thrombocytopenia that was associated with its use (22) it is now rarely employed in clinical medicine. Gangarosa, Johnson, and Ramos were the first to report that ristocetin caused microscopic platelet agglutination and lysis in vitro (22). Howard and Firkin extended these observations and further demonstrated that at concentrations in excess of 2 mg/ml ristocetin also precipitates fibrinogen (9). The results of the present study are in general agreement with those of Howard and Firkin and indicate that human platelets in citrated platelet-rich plasma are aggregated by ristocetin in concentrations, ranging from 1.0–1.5 mg/ml, that do not precipitate fibrinogen.

The observation that ristocetin did not release LDH, a cytoplasmic enzyme (23), indicates that under the conditions of this study, the antibiotic does not produce platelet lysis. The type of platelet aggregation produced by ristocetin has some features that are similar to those associated with ADP-induced aggregation. For example, ristocetin-induced aggregation is immediate and is inhibited by adenosine and by combined inhibition of both respiration and glycolysis (24). However, aggregation was only partially inhibited by a concentration of EDTA that completely blocks ADP-induced aggregation; conversely, it was totally blocked by a concentration of KCN that only partially inhibited ADP-induced aggregation, in confirmation of previous observations of Howard and Firkin (9). In addition, ristocetin produced a small amount of platelet aggregation in patients with Glanzmann's thrombasthenia, in contrast to the total absence of ADP-induced aggregation in this disorder (10). Unlike collagen-induced platelet aggregation, the release reaction appears to play only a minor role in ristocetin-induced aggregation, particularly in its early phase. For example, a significant amount of aggregation, occurring within 2 min of the addition of ristocetin, was associated with only a minimal release of either platelet ADP or serotonin. Substantial amounts of ADP (and relatively lesser amounts of serotonin) were released during a later stage of platelet aggregation; this released ADP may play some role in ristocetin-induced aggregation. In fur-

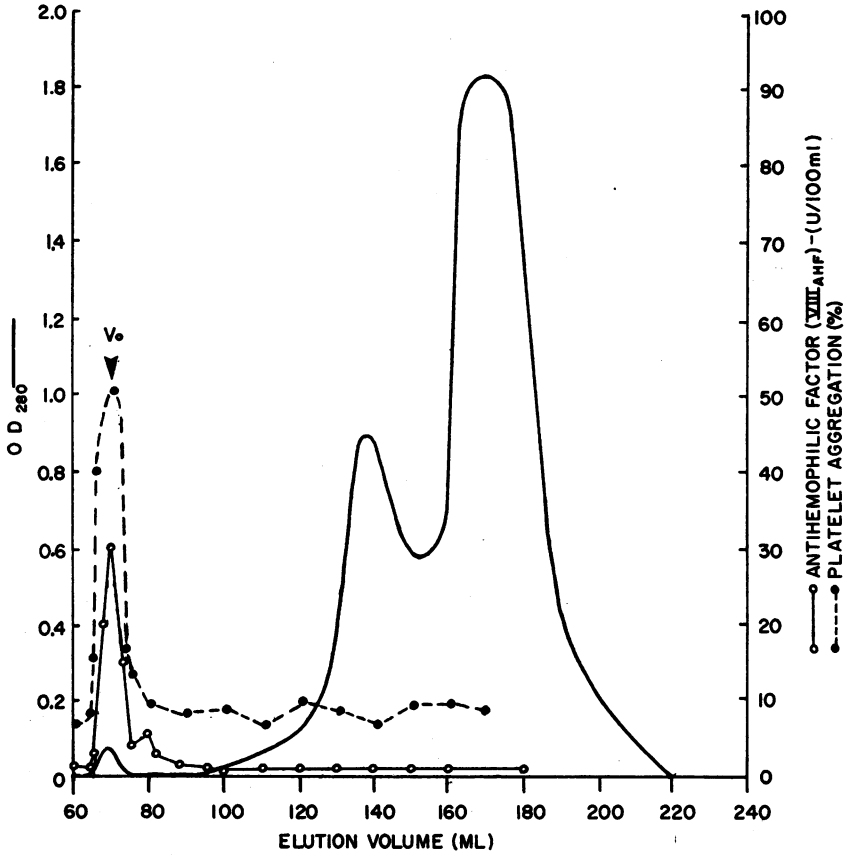

FIGURE 4 Chromatography of normal cryoprecipitate. Cryoprecipitate obtained from 40 ml of normal plasma was chromatographed on Bio-Gel 5M and the fractions were analyzed for protein (OD_{280}), $VIII_{AHF}$ activity and their ability to correct the abnormality of ristocetin-induced platelet aggregation in a patient with von Willebrand's disease. The latter (von Willebrand factor activity) was studied by adding a 0.5 ml aliquot of the column fractions to 1.4 ml of the patient's platelet-rich plasma before determination of ristocetin-induced aggregation. Note that $VIII_{AHF}$ and von Willebrand factor activity were both found in the void volume (V_o).

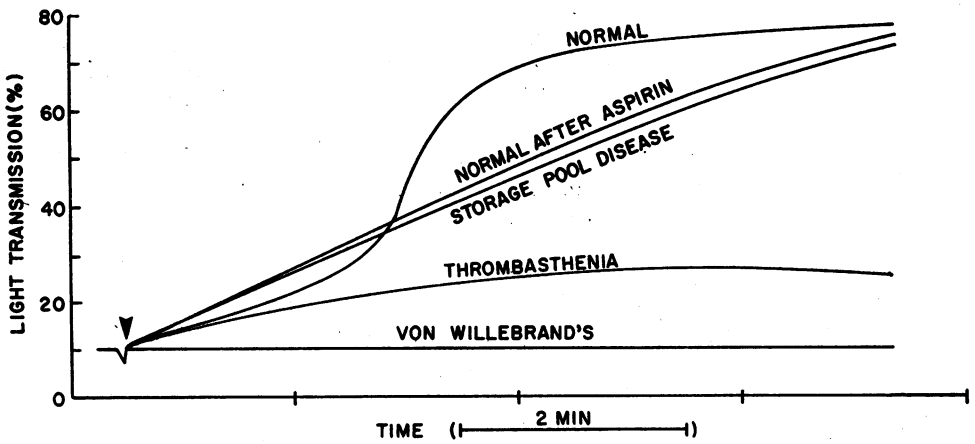

FIGURE 5 Ristocetin-induced platelet aggregation in other conditions associated with platelet dysfunction. Ristocetin (1.2 mg/ml) was added to platelet-rich plasma. Changes in light transmission shown are diagramatic.

TABLE IV

Specific Correction of Abnormal Ristocetin-Induced Aggregation by Factor VIII, Unrelated to $VIII_{AHF}$ Activity

Substance added to von Willebrand PRP				Ristocetin-induced* platelet aggregation
Subject source	Substance	Treatment of substance at 37°C, ½ h	$VIII_{AHF}$	
			U/100 ml	%
—	Buffer	—	0	0
Normal				
	Plasma	Saline	110	86
	Plasma	Rabbit anti-VIII	7	4
	Plasma	Human anti-VIII	2.5	87
	HMW fraction‡	Saline	30	83
	HMW fraction	Rabbit anti-VIII	<1	7
	HMW fraction	Human anti-VIII	<1	81
Hemophiliac				
	Plasma	Saline	<1	83
	Plasma	Rabbit anti-VIII	<1	0
	Plasma	Human anti-VIII	<1	82
	HMW fraction‡	—	<1	72
von Willebrand's				
	Plasma	Saline	2	0
	HMW fraction‡	—	<1	6

* Table represents a composite of four experiments in which 0.5 ml of the substances indicated (treated as shown) were added to 1.4 ml of PRP of either of two patients with severe von Willebrand's disease.

‡ High molecular weight (HMW) fraction is the fraction with the highest OD reading which was eluted in the void volume after chromatography of 40 ml of cryoprecipitate on Bio-Gel 5M, similar to the experiment shown in Fig. 4. In normal cryoprecipitate, this fraction coincided with the peak of the $VIII_{AHF}$ activity.

ther support of this possibility was the finding that the rate of platelet aggregation during the rapid phase of ristocetin-induced aggregation was somewhat decreased in normal subjects in whom the release mechanism had been inhibited by aspirin ingestion (13) and in patients whose platelets are deficient in the storage-pool of ADP (11, 12). In neither case, however, was this decrease sufficient to reduce the total amount of platelet aggregation that eventually occurred. The mechanism by which ristocetin aggregates platelets is not yet known. Previous studies have shown that ristocetin inhibits the synthesis of peptidoglycans in bacterial cell walls either by steric interference with their polymerization or by forming stable complexes with mucopeptide precursors (25–27). Whether similar interactions with the platelet surface are involved in ristocetin-induced platelet aggregation remains to be determined.

Ristocetin-induced platelet aggregation was decreased in 10 patients with von Willebrand's disease; this confirms the previous findings of Howard and Firkin, who reported decreased aggregation in two out of three patients with this disorder (9). In a more recent paper, these authors have reported decreased aggregation in 9 of 14 patients with von Willebrand's disease (28) and suggest that patients with normal ristocetin-induced platelet aggregation either have a different abnormality or a lesser degree of severity of the basic abnormality. Although the evidence is not conclusive, we are inclined at present to favor the latter hypothesis. Previous studies have shown that platelet aggregation by other substances such as collagen, ADP, and epinephrine is normal in von Willebrand's disease (1). The impaired aggregation of platelets by ristocetin, and by no other substance, appeared to be unique among the patients with congenital bleeding disorders studied to those with von Willebrand's disease. The conditions of this test, like those of the glass bead retention tests, are remote from those that occur during hemostasis. Nevertheless, there was a good correlation between the degree of abnormality of the bleeding time and $VIII_{AHF}$ level on the one hand and the magnitude of the defect in ristocetin-induced aggregation on the other. This correlation suggests that the prolonged bleeding time in von Willebrand's disease is somehow related to a defect in the formation of a hemo-

static platelet aggregate during the primary arrest of the bleeding. Baumgartner has developed and described a method for studying the adhesion of platelets in flowing blood to the subendothelium of rabbit aorta (*29*), and, using this method, we have recently found a significant defect of platelet adhesion in five patients with von Willebrand's disease (30). Since the interaction of platelets with collagen appears to be normal in von Willebrand's disease (1), these findings suggest that their nonadhesion to some other vascular component may account for the hemostatic defect in this disorder. How this may be related to an abnormality in ristocetin-induced platelet aggregation is not clear. Possibly, similar functional groups on the ristocetin molecule and vascular components may be involved.

The present study lends further support to the possibility suggested in previous studies that the defect in hemostasis and platelet function in von Willebrand's disease is not owing to an intrinsic platelet defect but rather to a deficiency of a plasma factor necessary for normal platelet function (3, 4, 7, 8). The abnormality of ristocetin-induced platelet aggregation in all patients was corrected by the addition of normal plasma. This correction factor eluted in the void volume with $VIII_{AHF}$ procoagulant activity after chromatography on Bio-Gel 5M, and its ability to correct the defect in platelet aggregation in von Willebrand's disease was destroyed by a rabbit antibody to human factor VIII. These findings suggest that the correction factor (von Willebrand factor, VWF) may be associated with the factor VIII molecule ($VIII_{vWF}$). The location of $VIII_{vWF}$ activity appears to reside on a different site on the factor VIII molecule than that determining $VIII_{AHF}$ activity. For example, the ability of normal plasma or factor VIII to correct the platelet aggregation defect in von Willebrand's disease was not affected by incubation with plasma from a hemophiliac with an inhibitor to $VIII_{AHF}$. In addition, correction activity was present in hemophilic plasma and in a high molecular weight ($> 5 \times 10^6$) fraction obtained from hemophilic cryoprecipitate. Unlike $VIII_{AHF}$ activity, correction factor activity was also present in serum. The findings suggest that the factor VIII molecule may contain at least two sites that are important in normal hemostasis. One of these determines $VIII_{AHF}$ procoagulant activity, while another determines an activity necessary for normal platelet function (the von Willebrand factor, $VIII_{vWF}$). Both activities appear to be decreased in von Willebrand's disease. In addition, patients with von Willebrand's disease are deficient in factor VIII antigen ($VIII_{AGN}$) (31, 32). We have developed an assay for the von Willebrand factor based on observations that ristocetin aggregates washed, normal platelets but only in the presence of plasma or factor VIII (15). The quantitative relationships which were found between $VIII_{AHF}$, $VIII_{vWF}$, and $VIII_{AGN}$ in normal subjects and in patients with von Willebrand's disease are the subject of an accompanying paper (15).

Finally, the mechanisms whereby factor VIII, a glycoprotein (33) is necessary for ristocetin-induced aggregation requires clarification. At higher concentrations than those required to aggregate platelets, ristocetin precipitates fibrinogen. We studied the effects on platelets of polylysine, which also precipitates fibrinogen (34), and concanavalin A, which precipitates factor VIII and other glycoproteins (33). Platelet aggregation by these two substances was the same in patients with von Willebrand's disease as in normal subjects; the mechanisms involved in platelet aggregation by these agents and by ristocetin are apparently different. Further studies on the nature of the interaction that may occur among ristocetin, factor VIII and platelets may help to clarify the mechanisms involved in ristocetin-induced aggregation and to provide useful information about the hemostatic defect in von Willebrand's disease.

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